Molecular Mechanisms of Immune-Mediated Lysis of Murine Renal Cancer: Differential Contributions of Perforin-Dependent Versus Fas-Mediated Pathways in Lysis by NK and T Cells[1,2]

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Mice bearing the experimental murine renal cancer Renca can be successfully treated with some forms of immunotherapy. In the present study, we have investigated the molecular pathways used by NK and T cells to lyse Renca cells. Renca cells normally express low levels of Fas that can be substantially enhanced by either IFN-γ or TNF-α, and the combination of IFN-γ + TNF-α synergistically enhances cell-surface Fas expression. In addition, cells pretreated with IFN-γ and TNF-α are sensitive to lysis mediated by Fas ligand (FasL)-expressing hybridomas (dllS), cross-linking of anti-Fas Abs or soluble Fas (FasL). Lysis via Fas occurs by apoptosis, since Renca shows all the typical characteristics of apoptosis. No changes in levels of bcl-2 were observed after cytokine treatments. We also examined cell-mediated cytotoxic effects using activated NK cells and T cells from *gld* FasL-deficient mice, and perforin-deficient mice, as well as wild-type C57BL/6 and BALB/c mice. Interestingly, the granule-mediated pathway predominated in killing of Renca by activated NK cells, while the Fas/FasL pathway contributed significantly to cell-mediated killing of Renca by activated T cells. These results suggest that killing of Renca tumor cells by immune effector cells can occur by both granule and Fas-mediated cytotoxicity. However, for the Fas-mediated pathway to function, cell surface levels of Fas need to be increased beyond a critical threshold level by proinflammatory cytokines such as IFN-γ and TNF-α. *The Journal of Immunology,* **1998, 161: 3957–3965.**

The murine renal cancer Renca responds partially to biologic therapy approaches that contain the cytokines IL-2 or IL-12 (1–4). Although the nature of the biologic effects that are critical for cytokine-induced antitumor responses are not yet known, most mice that survive biological therapies display immune memory and resist tumor rechallenge (2, 5). Also, a large amount of experimental data support a critical role for the host's immune system, particularly $CD8^+$ T cells, for complete eradication of this tumor in vivo (6).

In recent years, it has become clear that NK cells and cytotoxic T cells can mediate their lytic effects through at least two distinct pathways including 1) the degranulation pathway that uses perforin, probably in combination with various granzymes (7–9), and 2) the Fas-based pathway in which interaction between Fas ligand (FasL)[4] expressed on the CTL and Fas on the target cell triggers apoptosis and cell death (10–12). Interaction between other members of the tumor necrosis family (TNF) expressed on effector cells with target cell surface TNFR family members also can result in target cell death (13, 14). The FasL-Fas interaction has been shown to play a very important role in limiting T cell numbers, since mutation of either ligand or receptor results in the lymphoproliferative diseases seen in *gld* and *lpr* mice (15, 16). However, the importance of FasL-Fas interactions in viral diseases or anti-tumor responses is less clear. It has been demonstrated that for some viral infections (particularly with noncytopathic viruses) the granule-mediated pathway is essential for viral clearance by cytotoxic cells (17). However, in other viral infections both perforin and Fas-dependent processes were utilized for viral clearance by $CD8^+$ T cells (18).

Several tumors express Fas in vitro and are sensitive to Fas-mediated killing by anti-Fas Abs (19–21). However, the relative contribution of Fas-mediated killing of tumor cells by cytotoxic immune effector cells is not well documented. Interestingly, not all tumors that express high levels of Fas on their surface are susceptible to Fas-mediated killing, suggesting that this lytic mechanism will be limited by characteristics of the specific tumor target cell (22, 23). Studies on tumor surveillance in perforin-deficient (P^0)

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[4] Abbreviations used in this paper: FasL, Fas ligand; [^{111}In]Ox, [^{111}indium]-oxine; ZVAD-FMK, Z-Val-Ala-Asp(OMe)-CH_2F; ZFA-FMK, Z-Phe-Ala-(OMe)-CH_2F; sFasL, soluble Fas ligand; PE, phycoerythrin; DAPI, 4,6-diamino-2-phenylindole; NWNA, nylon wool nonadherent; P^0, perforin deficient; TNF, tumor necrosis family.

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mice demonstrated that perforin-dependent cytotoxicity was a crucial mechanism of resistance against injected tumor cell lines as well as viral and chemical carcinogenesis, whereas Fas provided only a minor contribution (24). However, most of the tumors used in this previous study were intrinsically resistant to Fas-mediated killing. Xenotransplantation of human tumors into nude mice also has convincingly demonstrated that cell-mediated cytotoxicity and antitumor efficacy predominantly involved granule-mediated killing (25, 26). Nonetheless, in such experimental systems, the species-specific activities of some cytokines and the strength of xenogeneic immune responses may minimize a role for cytokines and Fas-mediated effects. Interestingly, loss of Fas accelerates lymphomogenesis in Eμ L-MYC transgenic mice (27). We have therefore used the syngeneic murine renal cancer Renca as a model to investigate the possible role of the granule-mediated vs Fas-mediated pathways in tumor cell lysis. In addition we have compared the relative contributions of these pathways in two types of cytotoxic lymphocytes, activated NK cells and T cells.

Materials and Methods

Mice

Specific pathogen-free BALB/c and C57BL/6 (B6) mice were obtained from the Animal Production area, National Cancer Institute, Frederick Cancer Research and Development Center (Frederick, MD). The B6Smn.C3H.FasL *gld* (B6-*gld*) and perforin-deficient (P^0) B6-Pfp^{tm1Sdz} mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred at our facility.

Tumor cell lines

The Renca tumor cell line is of BALB/c origin. The P815 mastocytoma (DBA/2) and the A20 B lymphoma (BALB/c) were all maintained in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 1× nonessential amino acids, 1 mM sodium pyruvate, penicillin (100 U/ml) and streptomycin (100 μg/ml), 10 mM HEPES, and 5×10^{-5}M 2-ME, pH 7.4 (complete medium). The A20 lymphoma is very sensitive to Fas-mediated lysis. The d11S hybridoma cells kindly provided by Dr. P. Henkart (National Cancer Institute, National Institutes of Health, Bethesda, MD) utilize FasL to mediate their cytotoxic activity.

Reagents

Mouse rIFN-γ (sp. act. 4.7×10^6 U/mg) was generously provided by Genentech (South San Francisco, CA). Mouse rTNF-α (10^7 U/mg) was purchased from PharMingen (San Diego, CA). Recombinant IFN-α A/D (10^7 U/mg) was kindly provided by Dr. Michael Brunda (Hoffmann-La Roche, Nutley, NJ). Anti-mouse Fas (Jo2) mAb and the hamster IgG isotype control were purchased from PharMingen. Soluble recombinant human FasL (sFasL) was purchased from Alexis (San Diego, CA). The enzyme inhibitors Z-Val-Ala-Asp-(OMe)-CH_2F (ZVAD-FMK) and Z-Phe-Ala-(OMe)-CH_2F (ZFA-FMK) were purchased from Enzyme Systems Products (Dublin, CA).

Flow cytometric analysis

Fas expression on Renca cells was monitored after incubation of 5×10^5 cells at 4°C for 20 min with phycoerythrin (PE)-labeled hamster anti-Fas Ab or PE-labeled hamster IgG isotype control Ab. After washing, cells were analyzed on a FACScan (Becton Dickinson, Mountain View, CA) flow cytometer using CELLQuest software. For phenotyping of effector cell subsets, cells were incubated at 4°C for 30 min with FITC, biotin and/or PE-labeled Abs. Abs used were anti-CD3, anti-CD4, and anti-CD8 for T cells and DX5 for NK cell phenotyping. All Abs were purchased from PharMingen. Cells were washed with PBS containing 0.1% sodium azide and 0.1% BSA. Cells stained with biotinylated Abs were then incubated at 4°C for 20 min with avidin-PerCp (Becton Dickinson, San Jose, CA). Cells were analyzed on a Becton Dickinson FACSort using Lysys software.

Northern blotting

Renca cells were cultured overnight in medium in the presence or absence of various cytokines. Cells were then trypsinized, washed in a large volume of ice-cold PBS, counted, and pelleted. Cells were resuspended in Trizol reagent (Life Technologies, Gaithersburg, MD) and total RNA was isolated for Northern analysis; 20 μg RNA was subjected to electrophoresis on a 1.2% agarose:formaldehyde gel and was transferred to Nytran. Blots were then hybridized with cDNA for murine Fas (kindly provided by Dr. S. Nagata, Osaka, Japan) and human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) (Clontech, Palo Alto, CA). Blots were washed twice for 15 min at 42°C in 2 × SSC/0.1% SDS and then twice for 15 min at 60°C in 0.2× SSC/0.1% SDS. The blots were then exposed to Kodak X-OMAT AR film for 1 to 3 days.

Cytotoxicity assays

Renca cells that had been incubated overnight in the presence or absence of various cytokines were labeled with ^{111}indium-oxine ([^{111}In]Ox) (Medi-Physics, Silver Spring, MD) as previously described (28). Briefly 1×10^6 target cells were incubated with 10 μCi of [^{111}In]Ox for 30 min at room temperature. Cells were then washed twice in complete medium and labeled cells (1×10^4) were then incubated for 18 h at 37°C in the presence or absence of various Abs or cells in a final volume of 200 μl; other target cells used were labeled in a similar manner. The d11S cells (FasL-positive hybridoma), activated NK cells, or T cells were added at various E:T ratios. Different concentrations of anti-Fas Ab (Jo2) or isotype control Ab were added alone or in the presence of P815 cells (1×10^5) to promote Ab cross-linking. This method of efficiently cross-linking Abs on target cells was kindly provided by Dr. H. Kojima, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD. Controls were always run with P815 cells in the absence of Abs. sFasL was added at various concentrations. In experiments in which inhibitors were used, the caspase inhibitors ZVAD-FMK or control ZFA-FMK (Enzyme System Products, Dublin, CA) were added to target cells at various concentrations in serum-free medium (100 μl) for 1 h prior to addition of the effectors in complete medium, followed by an 18-h incubation at 37°C. After overnight incubation, supernatants were harvested and counted on a gamma counter. Specific killing (% cytotoxicity) was calculated as [(experimental release − spontaneous release) ÷ (maximal release − spontaneous release)] × 100. All groups were run in triplicate, and all experiments were performed three or more times with similar findings. Student's *t* test was used to determine the significance of cytotoxicity differences between groups.

Staining of DNA

Renca cells were grown on 22-mm × 22-mm coverslips in the presence or absence of cytokines overnight and then treated with anti-Fas or isotype control Ab at 1 μg/ml for a further 16 h. Cells were then fixed in situ by the addition of 0.2 ml of 37% formaldehyde to wells containing 2 ml of medium for 10 min at room temperature. The cells were then treated with 1 ml of PBS containing 0.2% Nonidet P-40 (Sigma, St. Louis, MO) for 2 min at room temperature and then rinsed once in PBS. The apoptotic cells were distinguished by staining of nuclear DNA with 4,6-diamino-2-phenylindole (DAPI) (Calbiochem, LaJolla, CA). DAPI was added at a concentration of 0.2 ng/ml of PBS and incubated at room temperature for 20 min. Cells were rinsed twice in PBS and the coverslips were maintained cell side down on a microscope slide using Vectashield Mounting Medium (Vector, Burlingame, CA). Nail polish was used to seal the coverslips to the slide to prevent drying. The cells were analyzed using a Zeiss Axiovert 135M microscope (Carl Zeiss, Thornwood, NY). Photographs were taken using Kodak Gold Max film.

Activated NK cells and T cells

Murine splenic NK cells were obtained using nylon wool nonadherent (NWNA) spleen cells. Briefly, NWNA cells were cultured for 3 days in complete medium containing 1000 U/ml of IL-2 (Cetus, Emeryville, CA). On day 3 the nonadherent cells were carefully removed and the adherent cells were cultured in medium containing IL-2 for a further 2 days. These cells were used as activated NK cells for cytotoxicity assays. By FACS analysis they were 40 to 60% $DX5^+$, 0 to 5% $CD3^+$ in all strains of mice used, the remaining cells being B cells. Activated murine T cells were prepared as previously described (29, 30). Briefly, resting mouse lymph node cells were cultured in 5 μg/ml Con A for 48 h, incubated with 10 mg/ml α-methylmannoside (Sigma) for 30 min at 37°C, washed, and incubated with 100 U/ml of IL-2 for a further 48 h. These cells were used as activated T cells in cytotoxicity assays and were >93% $CD3^+$ <2% $CD3^-$, $DX5^+$ from all strains of mice used. Activated T cells were used as

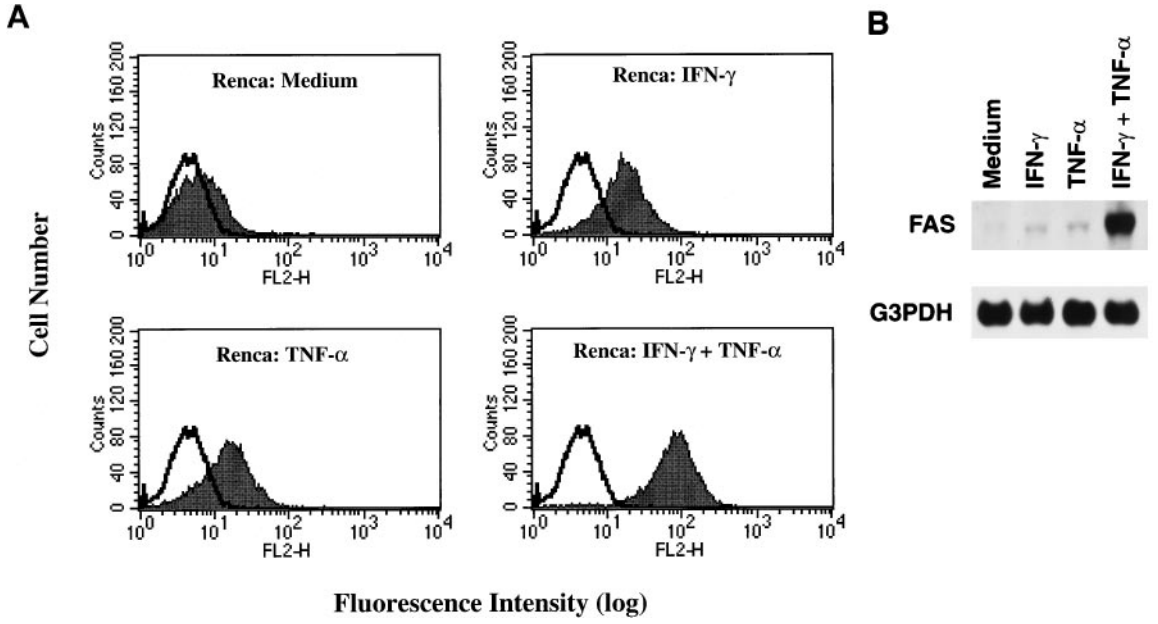

FIGURE 1. Expression of Fas on Renca. Renca cells were treated overnight with medium, IFN-γ (100 U/ml), TNF-α (100 U/ml), or a combination of IFN-γ and TNF-α. *A*, Expression of Fas was analyzed by flow cytometry. The dark shaded histograms represent immunostaining with an anti-Fas Ab (Jo2) and light shaded histograms represent staining with isotype-matched control Ab. *B*, RNA from Renca cells was subjected to Northern analysis using labeled Fas and G3PDH cDNAs as probes.

effectors in the presence of an Ab to mouse CD3 (PharMingen) at 1 μg/ml to promote cross-linking of the TCR. Appropriate control Ab was added at the same concentration.

Results

Expression of Fas on Renca is regulated by cytokines

Renca cells expressed low but detectable levels of Fas on their cell surface in vitro. However, treatment of cells overnight with either IFN-γ or TNF-α resulted in significant increases in Fas expression (Fig. 1*A*). The levels of induction by individual cytokines were approximately equivalent. Interestingly, the combination of these two cytokines synergistically enhanced Fas expression on Renca. Concentrations of IFN-γ as low as 1 U/ml could synergistically interact with TNF-α to give maximal Fas expression (data not shown). Northern blot analysis also revealed a significant increase of Fas mRNA on treatment with cytokine combinations (Fig. 1*B*).

Effects of increases in Fas expression on sensitivity to Fas-mediated killing

The effect of increases on Fas expression on susceptibility of Renca to killing through FasL-Fas interaction was tested using a variety of approaches. Methods of target cell stimulation through Fas induced a similar pattern of response. Untreated Renca cells (low Fas) and cells treated with individual cytokines (intermediate Fas) were resistant to Fas-mediated killing. However, Renca cells treated with a combination of IFN-γ and TNF-α (high Fas) were efficiently killed by d11S cells, cross-linking of anti-Fas Abs (by P815 cells), and sFasL (Fig. 2). The levels of killing by soluble anti-Fas Abs alone were somewhat variable between experiments (5–40% lysis) probably since efficient cross-linking of cell surface Fas is necessary for optimal killing of Renca (data not shown).

Molecular mechanisms of Fas-mediated killing of Renca

The [^{111}In]Ox release assay used to assess cytotoxicity does not distinguish between apoptotic or necrotic death of tumor cells. We therefore examined whether Fas engagement on cytokine-treated Renca cells triggered apoptosis. Microscopic observation of cells suggested that Renca cells were dying due to apoptosis since many of the morphologic characteristics of apoptosis (cell shrinkage, chromatin condensation, membrane blebbing, formation of apoptotic bodies, and loss of adherence) were observed (data not shown). Further, staining of nuclei with DAPI demonstrated DNA condensation and nucleosomal fragmentation consistent with apoptotic death occurring only in Renca cells treated with combinations of cytokines and anti-Fas Abs (Fig. 3). Fragmentation of Renca DNA, as determined by propiduim iodide staining of nuclei, also demonstrated a pattern typical of apoptosis only in cells treated with both cytokines and exposed to anti-Fas Abs (data not shown). Preincubation of cytokine-treated Renca cells with the caspase inhibitor ZVAD-FMK completely abolished [^{111}In]Ox release in response to d11S cells (Fig. 4), and a lack of apoptotic nuclei were seen in corresponding experiments with DAPI staining (data not shown). This is consistent with Fas-mediated apoptosis of Renca being caused by the caspase family of proteases.

In contrast, cytokine treatment of Renca did not effect expression of the anti-apoptotic factor bcl-2 as assessed by Western blotting (data not shown) suggesting that modulation of bcl-2 did not play a major role in regulating Fas-mediated apoptosis of Renca. Furthermore, the apoptotic killing of Renca induced by treatment with staurosporine was only slightly increased by pretreatment of cells with IFN-γ and TNF-α (Fig. 5*A*). This argues against potent effects of this cytokine combination on the apoptosis of Renca per se, and is more consistent with the effects of these cytokines being limited to Fas-mediated apoptosis. Although cross-linking of Fas could mediate significant killing of cytokine-treated Renca cells, TNF-α showed little to no killing activity (Fig. 5*B*). Also, TNF-α did not further enhance Fas-mediated cytotoxicity (Fig. 5*C*). It should be noted that even in combination with cycloheximide, TNF-α could not trigger significant killing of Renca in the presence or absence of cytokine pretreatment (data not shown). This suggests that the TNF-α pathway for inducing apoptosis in Renca is probably nonfunctional.

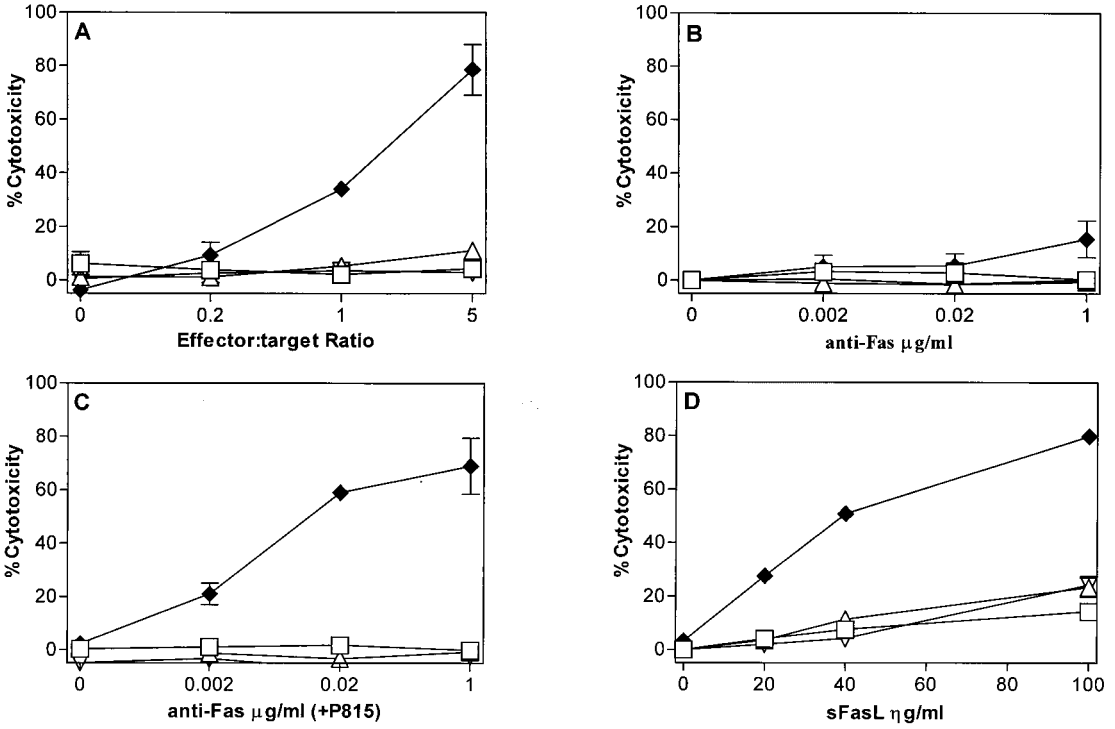

FIGURE 2. Fas-mediated cytotoxicity of Renca. Renca cells treated overnight with medium (□), IFN-γ (100 U/ml) (△), TNF-α (100 U/ml) (▽), or a combination of IFN-γ and TNF-α (◆) were used as target cells in an [^{111}In]Ox release assay. Effectors were d11S hybridoma cells (*A*), anti-Fas Ab (*B*), anti-Fas Ab plus P815 cells to promote cross-linking (*C*), and soluble recombinant human Fas L (*D*). After 18-h incubation cell supernatants were harvested and counted.

Cell-mediated lysis of Renca

Although the preceding results demonstrated that cytokine-treated Renca cells can be killed via the Fas pathway, the relative importance of this pathway to cell-mediated killing of Renca is not clear. We therefore examined lysis of Renca by two different types of cytolytic effector cells, activated NK cells and T cells. Furthermore, we used FasL-deficient (*gld*) or P^0 effector cells, as well as effectors from wild-type mice, in order to demonstrate the relative contributions of granule and Fas-mediated cytotoxicities. Killing of Renca by activated NK cells was predominantly performed by granule-mediated killing (Fig. 6). P^0 NK cells displayed negligible cytotoxic activity against Renca as well as a variety of other target cells, including A20. Interestingly, some killing was observed when P^0 NK cells were used at higher E:T ratios against cytokine-treated Renca (data not shown), suggesting FasL could play a minor role in NK lysis. Also, killing by *gld* NK cells was only slightly reduced compared with wild-type controls. The NK-mediated killing occurred either in the presence or absence of prior cytokine treatment; indeed, killing of Renca targets by NK cells was usually reduced on cytokine treatment. Also, the killing by activated NK cells was not influenced by treatment of target cells with the caspase inhibitor ZVAD-FMK. These accumulated results are consistent with a minimal role for Fas-mediated killing of Renca by activated NK cells.

Interestingly, a somewhat different pattern was observed when analyzing killing of Renca by activated T cells (Fig. 7). T cell-mediated killing in vitro was significantly enhanced on triggering through cross-linking of the TCR, which is known to be necessary for optimal up-regulation of cell surface FasL (31, 32). Lysis by BALB/c and B6 T cells was equivalent after TcR cross-linking, indicating that both allelic forms of murine FasL (33) could efficiently kill Renca cells. Cytokine pretreatment of target cells was not necessary for lysis; however, it is likely that sufficient IFN-γ and TNF-α were produced by the activated T cells during the course of the assay to up-regulate target cell Fas expression. Supernatants from incubations of anti-CD3 activated T cells and Renca targets had easily detectable levels of IFN-γ (>3000 pg/ml) and TNF-α (>20 U/ml). A comparison of Renca killing by activated T cells of various strains showed that P^0 T cells kill at levels comparable with T cells from wild-type mice, suggesting that killing is granule independent. In contrast, there was always a significant decrease in killing by *gld* T cells that was particularly evident on Renca targets (50–75% decrease). Similar observations have been made in four independent experiments. However, it is important to note that although killing by *gld* T cells was reduced, it was not completely abolished. If granules are not involved, this suggests a third effector pathway for the lysis of Renca cells. Attempts were made to further elucidate the mechanisms contributing to T cell-mediated lysis of Renca by examining effects of the caspase inhibitor ZVAD-FMK. As seen in Figure 8, killing mediated by *gld* and BALB/c T cells could be significantly reduced by pretreatment of target cells with ZVAD-FMK. This contrasts with lysis mediated by NK cells (which is predominantly granule mediated) in which ZVAD-FMK treatment had no effect. This finding suggests that for most of the T cell-mediated killing, activation of caspase in the target cells was essential for lysis, and further reinforced the mechanistic differences between NK and T cell-mediated lysis. Finally, lysis mediated by *gld* T cells was also substantially reduced by ZVAD-FMK, suggesting that the residual lysis observed with these T cells was neither due to granule-mediated nor Fas-mediated killing, but rather to another caspase-dependent cytotoxic event induced by T cells or their products.

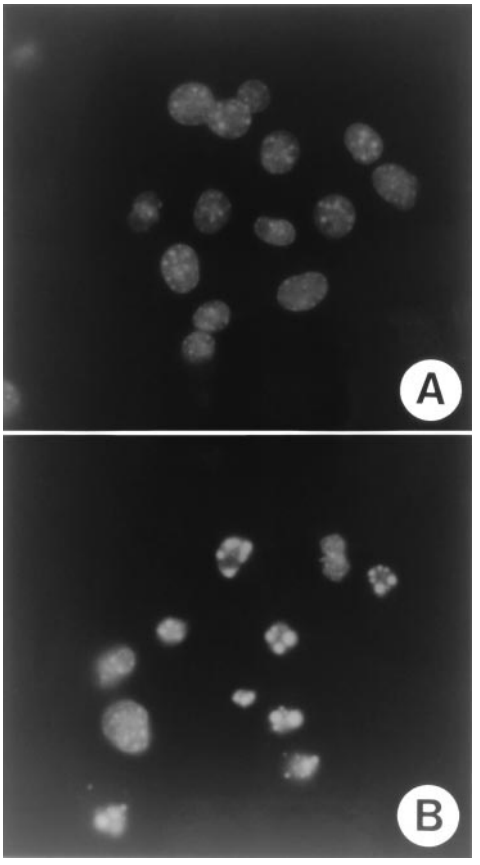

FIGURE 3. Fas-mediated lysis of Renca is due to apoptosis. Renca cells were treated overnight with medium (*A*) or IFN-γ (100 U/ml) and TNF-α (100 U/ml) (*B*). Anti-Fas Ab (Jo2) was then added at 1 μg/ml and cells were incubated for a further 18 h. Cells were then fixed and nuclei were stained with DAPI as described in *Materials and Methods*.

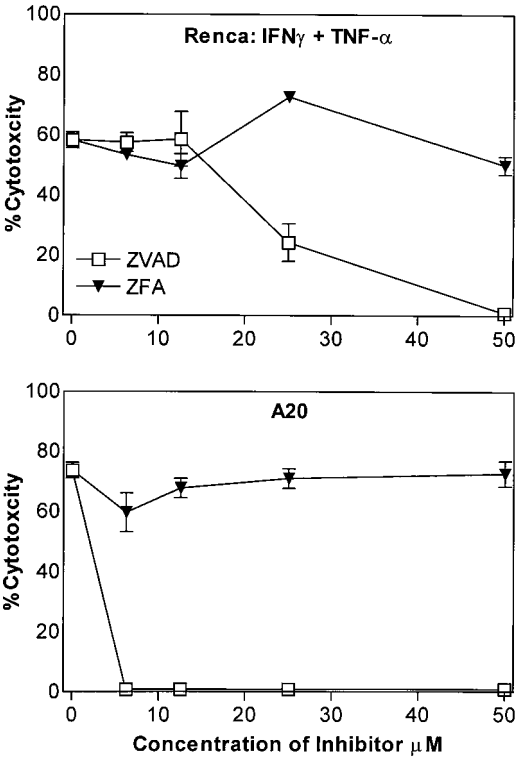

FIGURE 4. Effects of enzyme inhibitors on Fas-mediated lysis of Renca. [^{111}In]Ox-labeled Renca (pretreated with IFN-γ and TNF-α) and A20 cells were treated with various concentrations of the protease inhibitors ZFA-FMK (▼) or ZVAD-FMK (□). The d11S effector cells were then added at an E:T ratio of 5:1 and incubations were continued for 18 h. Supernatants were then harvested and counted.

Discussion

In recent years much interest has focused on apoptosis as a mechanism to control cell numbers (34, 35). The interaction between FasL and Fas are biologically important in maintaining lymphoid cell numbers since the *lpr* and *gld* mutant mice show extensive lymphoid proliferation (15, 16). However, it is likely that FasL-Fas interaction also plays a role in other important biologic processes. Many cells in the body constitutively express Fas, or can be induced to do so (36). In contrast, FasL expression is usually limited to activated T cells, NK cells, and cells of certain immunologically privileged sites such as testis and eye (37, 38). FasL-bearing cells contribute a second cytolytic mechanism in addition to granule-mediated cytotoxicity whereby effector cells can lyse infected or neoplastic Fas-expressing targets (8, 39). This may be important in elimination of certain viruses (18), however, the potential for lysis of "innocent bystander" cells expressing Fas also may be the basis for some autoimmune pathologies (40–44).

The role of FasL-mediated lysis in the therapy of solid tumors is not well characterized. Some reports have described the presence or induction of Fas on the surface of osteosarcoma cells, human glioma cells, and renal cancer cells by various cytokines in vitro (20, 21, 45). With Renca cells we found a substantial synergy between IFN-γ and TNF-α for the induction of Fas expression. Since IFN-γ and TNF-α are thought to mediate their effects through different signaling pathways (46, 47), some components of these pathways must collaborate for increased transcription of the Fas gene in Renca. Previous studies on a number of human renal cancer cell lines demonstrate that most constitutively express Fas on their cell surface and this expression could be moderately upregulated by IFN-γ used as a single agent (21). In Renca, the sensitivity to Fas-mediated lysis correlated very well with levels of cell surface Fas. The simplest explanation for this would be that quantitative increases in Fas expression above a certain threshold level would result in a signaling intensity that would promote apoptosis. It could be argued that cytokine treatments could also enhance pro-apoptotic factors or decrease levels of anti-apoptotic factors, thereby amplifying apoptosis. However, Renca cells transfected with Fas and selected for high expression are very sensitive to Fas-mediated apoptosis even in the complete absence of prior cytokine treatment. In these studies, RNase protection assays did not demonstrate major changes in the levels of a range of pro- and anti-apoptotic factors, at least at the level of transcription (J.-K. Lee, T. J. Sayers, A. D. Brooks, T. C. Back, J. M. Wigginton, and R. H. Wiltrout, unpublished data). In addition, we observed no changes in bcl-2 at the protein level after cytokine treatment. Furthermore, apoptosis mediated by another stimulus (staurosporine) was not affected substantially by prior cytokine treatment of Renca. Taken together, these data suggest it is unlikely that modification of components of the "death machinery" plays a critical role in Renca apoptosis. However, the level of cell surface Fas expression by Renca may ultimately be the critical factor that controls whether Renca cells survive or die following exposure to FasL.

The coupling of the Fas signal to the death pathway involves receptor trimerization followed by the binding of adaptor proteins like FADD (48, 49). Subsequently, FADD recruits FLICE through homotypic interaction between "death effector domains" on each

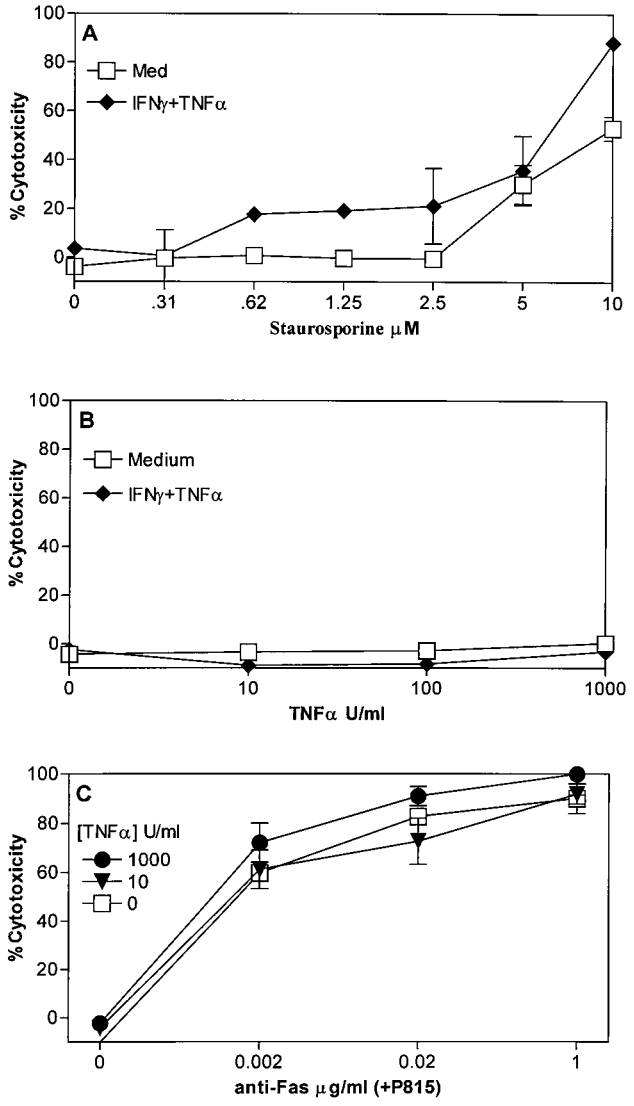

FIGURE 5. Effects of staurosporine and TNF-α on lysis of Renca. (*A*, Renca cells incubated overnight with medium (□) or IFN-γ (100 U/ml) plus TNF-α (100 U/ml) (◆) were washed and labeled with [^{111}In]Ox. Various concentrations of staurosporine were then added and 18 h later cell supernatants were harvested and counted. (*B*, Renca cells incubated overnight with medium (□) or IFN-γ (100 U/ml) plus TNF-α (100 U/ml) (◆) were washed and labeled with] [^{111}In]Ox. TNF-α was added at various concentrations and 18 h later cell supernatants were harvested and counted. (*C*, Renca cells incubated overnight with IFN-γ (100 U/ml) plus TNF-α (100 U/ml) were washed and labeled with [^{111}In]Ox. Target cells were then incubated for 18 h with anti-Fas (□), anti-Fas plus TNF-α (1000 U/ml) (●), or anti-Fas plus TNF-α (10 U/ml) (▼) in the presence of P815 cells. Cell supernatants were then harvested and counted.

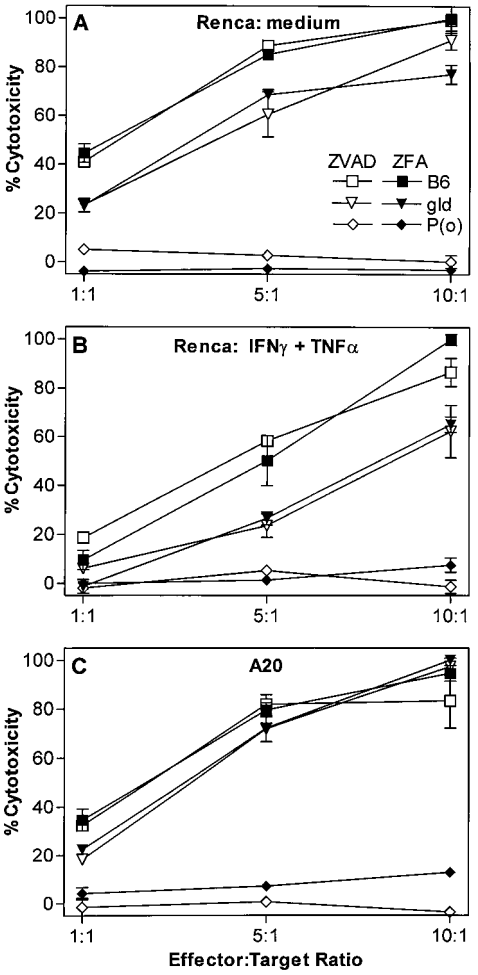

FIGURE 6. NK lysis of tumor target cells. Target cells *A*) Renca plus medium, *B*) Renca with IFN-γ (100 U/ml) plus TNF-α (100 U/ml) overnight, and *C*) A20 cells were labeled with [^{111}In]Ox, then incubated for 1 h in the presence of ZFA-FMK (closed symbols) or ZVAD-FMK (open symbols). Activated NK effector cells from B6 (squares), *gld*, (triangles), or P^0 (diamonds) were added to the targets at various E:T ratios for 18 h. Supernatants were then harvested and counted.

protein (50, 51). The resultant activation of the caspase domain of FLICE is then thought to trigger the caspase enzyme cascade, resulting ultimately in apoptosis. Our studies using the caspase inhibitor ZVAD-FMK show that this caspase activation also is critical for Fas-mediated apoptosis of Renca. Interestingly, no differences in transcription of FADD or FLICE have been observed in RNase protection assays on Renca cells treated with cytokines, further indicating that transcriptional activation of these factors is not critical to Renca apoptosis (J.-K. Lee, et al., unpublished data).

To establish a more biologically relevant model for Renca killing, we compared two cytotoxic cell populations, activated NK cells and activated T cells. On examining the cytotoxic effects of these populations, it was clear that Renca cells could be efficiently killed by cytotoxic cells using both perforin and Fas-mediated pathways. However, for activated NK cells, the dominant lytic mechanism was granule-mediated killing. This killing was not sensitive to the caspase inhibitor ZVAD-FMK. Therefore, although perforin can modulate localization of granzyme B in target cells (52), and granzyme B can activate caspases (53, 54), caspase activation is not an essential prerequisite for granule-mediated killing. Our data on NK killing are consistent with previous observations in which granule-mediated cytotoxicity was not blocked by caspase inhibitors (55). Conversely, when activated T cells were stimulated through the TCR, Fas-mediated killing was consistently responsible for a significant proportion of the lysis observed, since T cells from *gld* mice exhibited reduced cytotoxic effects whereas killing by P^0 T cells was at normal levels.

The protocol we used to generate activated T cells was based on previous procedures designed to study activation-induced cell death in T cells. In fact it is highly probable that (with the possible exception of *gld* T cells) some of the effector cells themselves are deleted by apoptosis during the period of the assay. Although killing of Renca by T cells from *gld* mice was reduced, it was not

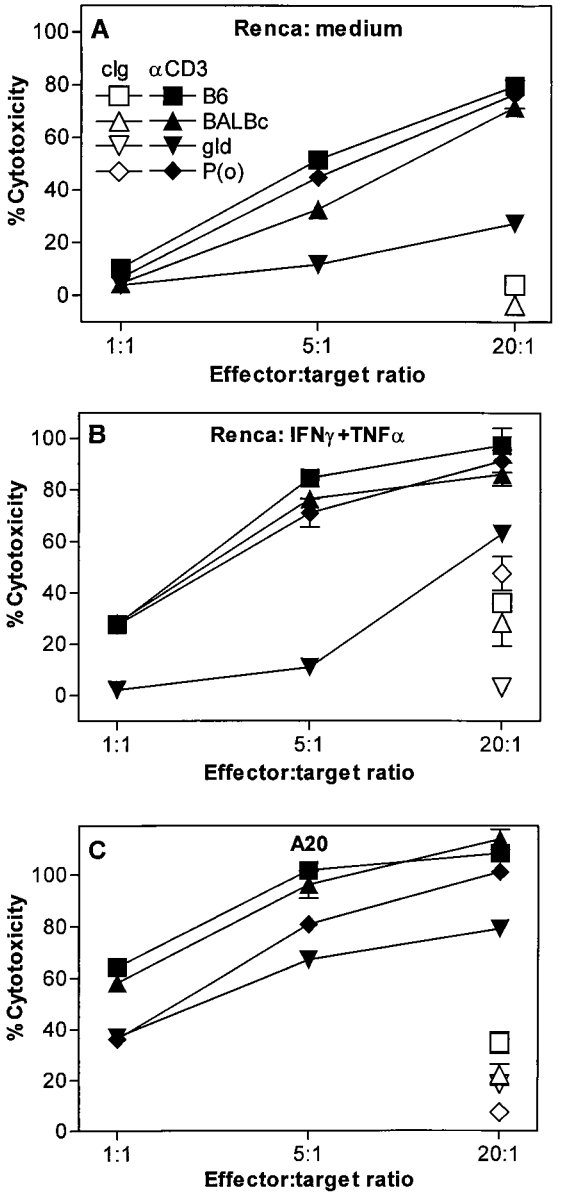

FIGURE 7. Activated T cell lysis of tumor target cells. Target cells *A*) Renca plus medium, *B*) Renca with IFN-γ (100 U/ml) plus TNF-α (100 U/ml) overnight, and *C*) A20 cells were labeled with [^{111}In]Ox. Effector cells from B6 (□), BALB/c (▲), *gld* (▼), and P^0 (◆) were added at various ratios in the presence of Ab to CD3 at 1 μg/ml, or at one ratio (20:1) in the presence of control Ab B6 (□), BALB/c (△), *gld* (▽), and P^0 (◇). After 18 h, cell supernatants were harvested and counted.

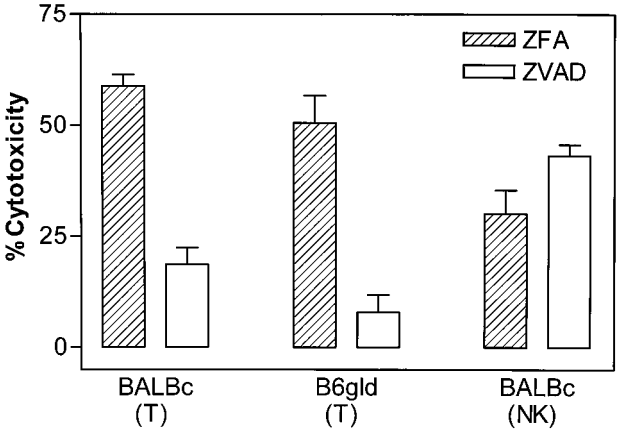

FIGURE 8. Effects of inhibitors on T cell lysis. Renca cells were incubated overnight with IFN-γ (100 U/ml) plus TNF-α (100 U/ml) then washed and labeled with [^{111}In]Ox. Enzyme inhibitors ZFA-FMK (▨) and ZVAD-FMK (□) were added at a concentration of 50 μM. Activated T cells from BALB/c and *gld* mice and activated NK cells from BALB/c mice were added at a 5:1 E:T ratio. After 18 h, cell supernatants were harvested and counted.

abolished, clearly indicating that T cells could induce target cell lysis by other mechanisms. Since lysis by *gld* T cells was still inhibited by ZVAD-FMK, it is likely that T cells can produce other factors that promote apoptosis. Although TNF-α did not seem to significantly contribute to Renca lysis, some contribution by other members of the TNF-TNFR families such as TRAIL (56) or TRANCE (57) cannot be ruled out. Another possibility is that T cells activated through their receptor also could utilize granule-mediated killing, yet the normal levels of killing by P^0 T cells argues against a significant contribution of granule-mediated cytotoxicity. The extent to which T cells used granule-mediated killing may vary depending upon their state of activation. If T cells were left in culture with IL-2 for a longer time period (>3 days), significant levels of lysis by T cells were observed in either the presence or absence of anti-CD3. Therefore, after more prolonged culture, T cells develop a lymphokine-activated killer phenotype in which cytotoxic activity is less dependent on activation through CD3 and granule-mediated killing plays a bigger role.

The differences noted between activated NK and T cell killing of Renca could therefore depend on the relative kinetics of activation of the granule-mediated pathway in T cells. NK cells are in effect "preactivated" with a full complement of perforin and granzymes (58–61). Therefore, upon appropriate recognition, they can immediately kill their targets with relatively rapid kinetics, using preformed perforin and granzymes. The strength and rapidity of this pathway may effectively mask weaker effects of FasL. It has been reported that highly purified murine NK cells can use FasL to mediate lysis (62). However, in these studies highly purified NK cells at very high E:T ratios were required for significant lysis of very susceptible Fas^+ targets. Therefore, although NK cells can probably use this pathway, it seems unlikely that it plays a major role in NK killing in vivo. In contrast to NK cells, resting T cells have very low levels of perforin and granzymes, which are increased during appropriate activation (63). If the ability to upregulate FasL in T cells appears prior to complete competence for granule-mediated killing, Fas-mediated apoptosis could play a significant role in T cell cytotoxicity against Fas-sensitive targets during early stages of T cell activation. We did not determine in this study which T cell subsets were responsible for the lysis observed. However, Fas-mediated lysis has been described for both $CD8^+$ and $CD4^+$ Th1 cells (64–66).

It is well established that T cells are critical for effective therapy of Renca. However, their precise role is unclear. It could be that their major importance is to produce various cytokines like IFN-γ, TNF-α, or IP-10 which can affect both tumor growth (2, 5, 67) and tumor vascularization (68, 69). However, eradication of preexisting tumor metastases is critical to effective therapy of this tumor. Successful immunotherapeutic regimens do result in the production of type I cytokines like IFN-γ and TNF-α (2, 67, 70). These cytokines (particularly IFN-γ) could have direct growth inhibitory effects on the tumor, increase class I and/or class II expression, promote cell-mediated immune responses, and significantly enhance tumor cell Fas expression. The ultimate contribution of various cell-mediated cytotoxic pathways to the immunotherapy of

Renca can be directly approached only after the *gld* mutation and perforin deficiency are transferred to mice with the same H-2^d BALB/c background of the Renca tumor. Such studies are currently underway. The overall implication of these studies is that cytokines produced during an immune response or immunotherapy could increase Fas expression on certain solid tumors, potentially sensitizing cells to FasL-mediated lysis. Activated immune effector cells capable of expressing FasL could then limit the growth and metastases of these tumors. Although ~20% of renal cancer patients respond to immunotherapy, the basis for these responses is unknown (71). An assessment of the possible role of FasL and related molecules in the immunotherapy of a variety of human renal cancers is therefore worthy of further study.

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References

1. Wiltrout, R. H., M. R. Boyd, T. C. Back, R. R. Salup, J. A. Arthur, and R. L. Hornung. 1988. Flavone-8-acetic acid augments systemic natural killer cell activity and synergizes with IL-2 for the treatment of murine renal cancer. *J. Immunol. 140:3261*.
2. Hornung, R. L., T. C. Back, D. S. Zaharko, W. J. Urba, D. L. Longo, and R. H. Wiltrout. 1988. Augmentation of natural killer cell activity, induction of IFN, and development of tumor immunity during the successful treatment of established murine renal cancer using flavone acetic acid and IL-2. *J. Immunol. 141:3671*.
3. Wigginton, J. W., K. L. Komschlies, T. C. Back, J. L. Franco, M. J. Brunda, and R. H. Wiltrout. 1996. Administration of interleukin 12 with pulse interleukin 2 and the rapid and complete eradication of murine renal cancer. *J. Natl. Cancer Inst. 88:38*.
4. Asano, T., A. Khanna, M. Lagman, B. Li, and M. Suthanthiran. 1997. Immunostimulatory therapy with anti-CD3 monoclonal antibodies and recombinant interleukin-2: heightened in vivo expression of mRNA encoding cytotoxic attack molecules and immunoregulatory cytokines and regression of murine renal cancer. *J. Urol. 157:2396*.
5. Sayers, T. J., T. A. Wiltrout, K. McCormick, C. Husted, and R. H. Wiltrout. 1990. Antitumor effects of α-interferon and γ-interferon on a murine renal cancer (Renca) in vitro and in vivo. *Cancer Res. 50:5414*.
6. Wiltrout, R. H. 1991. The immunotherapy of murine renal cancer. *Prog. Neuroendocrinimmunol. 4:154*.
7. Kägi, D., B. Ledermann, K. Burki, P. Seiler, B. Odermatt, K. J. Olsen, E. R. Podack, R. M. Zinkernagel, and H. Hengartner. 1994. Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. *Nature 369:31*.
8. Podack, E. R. 1995. Execution and suicide: cytotoxic lymphocytes enforce draconian laws through separate molecular pathways. *Curr. Opin. Immunol. 7:11*.
9. Liu, C., C. M. Walsh, and J. D-E. Young. 1995. Perforin: structure and function. *Immunol. Today 16:194*.
10. Nagata, S., and P. Golstein. 1995. The Fas death factor. *Science 267:1449*.
11. Berke, G. 1997. The Fas-based mechanism of lymphocytotoxicity. *Hum. Immunol. 54:1*.
12. Henkart, P. A. 1994. Lymphocyte-mediated cytotoxicity: two pathways and multiple effector molecules. *Immunity 1:343*.
13. Grell, M., P. H. Krammer, and P. Scheurich. 1994. Segregation of APO-1/Fas antigen- and tumor necrosis factor receptor-mediated apoptosis. *Eur. J. Immunol. 24:2563*.
14. Wong, G. H. W., and D. V. Goeddel. 1994. Fas antigen and p55 TNF receptor signal apoptosis through distinct pathways. *J. Immunol. 152:1751*.
15. Watanabe-Fukunga, R., C. I. Brannan, N. G. Copeland, N. A. Jenkins, and S. Nagata. 1992. Lymphoproliferative disorder in mice explained by defects in Fas antigen that mediates apoptosis. *Nature 356:314*.
16. Takahashi, T., M. Tanaka, C. I. Brannan, N. A. Jenkins, N. G. Copeland, T. Suda, and S. Nagata. 1994. Generalized lymphoproliferative disease in mice, caused by a point mutation in the Fas ligand. *Cell 76:969*.
17. Kägi, D., P. Seiler, J. Pavlovic, B. Ledermann, K. Bürki, R. M. Zinkernagel, and H. Hengartner. 1995. The roles of perforin- and Fas-dependent cytotoxicity in protection against cytopathic and noncytopathic viruses. *Eur. J. Immunol. 25:3256*.
18. Topham, D. J., R. A. Tripp, and P. C. Doherty. 1997. $CD8^+$ T cells clear influenza virus by perforin or Fas-dependent processes. *J. Immunol. 159:5197*.
19. Trauth, B. C., C. Klas, A. M. J. Peters, S. Matzuku, P. Möller, W. Falk, K. Debatin, and P. H. Krammer. 1989. Monoclonal antibody-mediated tumor regression by induction of apoptosis. *Science 245:301*.
20. Weller, M., K. Frei, P. Groscurth, P. H. Krammer, Y. Yonekawa, and A. Fontana. 1994. Anti-Fas/APO-1 antibody-mediated apoptosis of cultured human glioma cells: induction and modulation of sensitivity by cytokines. *J. Clin. Invest. 94:954*.
21. Nonomura, N., T. Miki, M. Yokoyama, T. Imazu, T. Takada, S. Takeuchi, N. Kanno, K. Nishimura, Y. Kojima, and A. Okuyama. 1996. Fas/APO-1-mediated apoptosis in human renal cell carcinoma. *Biochem. Biophys. Res. Commun. 229:945*.
22. Keane, M. M., S. A. Ettenberg, G. A. Lowery, E. K. Russell, and S. Lipkowicz. 1996. Fas expression and function in normal and malignant breast cell lines. *Cancer Res. 56:4791*.
23. Owen-Schaub, L. B., R. Radinsky, E. Kruzel, K. Berry, and S. Yonehara. 1994. Anti-Fas on nonhematopoetic tumors: levels of Fas/APO-1 and bcl-2 are not predictive of biological responsiveness. *Cancer Res. 54:1580*.
24. van den Broek, M. F., D. Kägi, F. Ossendorp, R. Toes, S. Vamvakas, W. K. Lutz, C. J. M. Melief, R. M. Zinkernagel, and H. Hengartner. 1996. Decreased tumor surveillance in perforin-deficient mice. *J. Exp. Med. 184:1781*.
25. Smyth, M. J., M. H. Kershaw, and J. A. Trapani. 1997. Xenospecific cytotoxic T lymphocytes: potent lysis in vitro and in vivo. *Transplantation 63:1171*.
26. Smyth, M. J., V. R. Sutton, M. H. Kershaw, and J. A. Trapani. 1996. Xenospecific cytotoxic T lymphocytes use the perforin- and Fas-mediated lytic pathways. *Transplantation 62:1529*.
27. Zornig, M., A. Grzeschiczek, M.-B. Kawalski, K.-U. Hartmann, and T. Moroy. 1995. Loss of Fas/Apo-1 receptor accelerates lymphomagenesis in Eu L-MYC transgenic mice but not in animals infected with MoMuLV. *Oncogene 10:2397*.
28. Wiltrout, R. H., D. Taramelli, and H. T. Holden. 1981. Measurement of macrophage-mediated cytotoxicity against adherent and non-adherent target cells by release of ^{111}indium-oxine. *J. Immunol. Methods 43:319*.
29. Zheng, L., G. Fisher, R. E. Miller, J. Pershon, D. H. Lynch, and M. J. Lenardo. 1995. Induction of apoptosis in mature T cells by tumor necrosis factor. *Nature 377:348*.
30. Hornung, F., L. Zheng, and M. J. Lenardo. 1997. Maintenance of clonotype specificity in CD95/Apo-1/Fas-mediated apoptosis of mature T lymphocytes. *J. Immunol. 159:3816*.
31. Vignaux, F., E. Vivier, B. Malissen, V. Depaetere, S. Nagata, and P. Golstein. 1995. TCR/CD3 coupling to Fas-based cytotoxicity. *J. Exp. Med. 181:781*.
32. Ju, S., D. J. Panka, H. Cui, R. Ettinger, M. El-Khatib, D. H. Sherr, B. Z. Stanger, and A. Marshak-Rothstein. 1995. Fas(CD95)/FasL interactions required for programmed cell death after T cell activation. *Nature 373:444*.
33. Kayagaki, N., N. Yamaguchi, F. Nagao, S. Matsuo, H. Maeda, K. Okumura, and H. Yagita. 1997. Polymorphism of murine Fas ligand that affects biological activity. *Proc. Natl. Acad. Sci. USA 94:3914*.
34. Raff, M. C. 1992. Social controls on cell survival and cell death. *Nature 356:397*.
35. Thompson, C. B. 1995. Apoptosis in the pathogenesis and treatment of disease. *Science 267:1456*.
36. Watanabe-Fukunaga, R., C. I. Brannan, N. Itoh, S. Yonehara, N. G. Copeland, N. A. Jenkins, and S. Nagata. 1992. The cDNA stucture, expression, and chromosome assignment of the mouse Fas antigen. *J. Immunol. 148:1274*.
37. Bellgrau, D., D. Gold, H. Selawry, J. Moore, A. Franzusoff, and R. C. Duke. 1995. A role for CD95 ligand in preventing graft rejection. *Nature 377:630*.
38. Griffith, T. S., T. Brunner, S. M. Fletcher, D. R. Green, and T. A. Ferguson. 1995. Fas ligand-induced apoptosis as a mechanism of immune privilege. *Science 270:1189*.
39. Nagata, S. 1997. Apoptosis by death factor. *Cell 88:355*.
40. Sabelko, K. A., K. A. Kelly, M. H. Cross, and J. H. Russell. 1997. Fas and Fas ligand enhance the pathogenesis of experimental allergic encephalomyelitis, but are not essential for immune privilege in the central nervous system. *J. Immunol. 159:3096*.
41. Waldner, H., R. A. Sobel, E. Howard, and V. K. Kuchroo. 1997. Fas- and FasL-deficient mice are resistant to induction of autoimmune encephalomyelitis. *J. Immunol. 159:3100*.
42. Bobé, P., D. Bonardelle, M. Reynès, F. Godeau, J. Mahiou, V. Joulin, and N. Kiger. 1997. Fas-mediated liver damage in MRL hematopoetic chimeras undergoing lpr-mediated graft-versus-host disease. *J. Immunol. 159:4197*.
43. Smyth, M. J. 1997. Fas ligand-mediated bystander lysis of syngeneic cells in response to an allogeneic stimulus. *J. Immunol. 158:5765*.
44. Ando, K., K. Hiroishi, T. Kanedo, T. Moriyama, Y. Muto, N. Kayagaki, H. Yagita, K. Okumura, and M. Imawari. 1997. Perforin, Fas/Fas ligand, and TNF-α pathways as specific and bystander killing mechanisms of hepatitis C virus-specific human CTL. *J. Immunol. 158:5283*.
45. Fellenberg, J., H. Mau, C. Scheuerflug, V. Ewerbeck, and K. Debatin. 1997. Modulation of resistance to anti-APO-1-induced apoptosis in osteosarcoma cells by cytokines. *Int. J. Cancer 72:536*.
46. Peska, S. 1997. The interferon receptors. *Semin. Oncol. 24:S9–18*.
47. Vandenabeele, P., W. Declercq, R. Beyaert, and W. Fiers. 1997. Two tumor necrosis factor receptors: structure and function. *Trends Cell Biol. 5:392*.
48. Chinnaiyan, A. M., K. O'Rourke, M. Tewari, and V. M. Dixit. 1995. FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. *Cell 81:505*.
49. Boldin, M. P., E. E. Varfolomeev, Z. Pancer, I. L. Mett, J. H. Chamonis, and D. Wallach. 1995. A novel protein that interacts with the death domain of Fas/APO1 contains a sequence motif related to the death domain. *J. Biol. Chem. 270:7795*.
50. Boldin, M. P., T. M. Goncharov, Y. V. Goltsev, and D. Wallach. 1996. Involvement of MACH, a novel MORT/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death. *Cell 85:803*.

51. Muzio, M., A. M. Chinnaiyan, F. C. Kischel, K. O'Rourke, A. Shevchenko, J. Ni, C. Scaffidi, J. D. Bretz, M. Zhang, R. Gentz, et al. 1996. FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing complex. *Cell 85:817*.
52. Shi, L., S. Mai, S. Israels, K. Browne, J. A. Trapani, and A. H. Greenberg. 1997. Granzyme B (GraB) autonomously crosses the cell membrane and perforin initiates apoptosis and GrzB nuclear localization. *J. Exp. Med. 185:855*.
53. Darmon, A. J., D. W. Nicolson, and R. C. Bleackley. 1995. Activation of the apoptotic protease CPP32 by cytotoxic T-cell-derived granzyme B. *Nature 377:446*.
54. Talanian, R. V., X. H. Yang, J. Turbov, P. Seth, T. Ghayur, C. A. Casiano, K. Orth, and C. J. Froelich. 1997. Granule-mediated killing: pathways for granzyme B-initiated apoptosis. *J. Exp. Med. 186:1323*.
55. Sarin, A., M. S. Williams, M. A. Alexander-Miller, J. A. Berzofsky, C. M. Zacharchuk, and P. A. Henkart. 1997. Target cell lysis by CTL granule exocytosis is independent of ICE/CED-3 family proteases. *Immunity 6:209*.
56. Wiley, S. R., K. Schooley, P. J. Smolak, W. S. Din, C. Huang, J. K. Nicholl, G. R. Sutherland, C. A. Smith, and R. G. Goodwin. 1995. Identification and characterization of a new member of the TNF family that induces apoptosis. *Immunity 3:673*.
57. Wong, B. R., J. Rho, J. Arron, E. Robinson, J. Orlinck, M. Chao, S. Kalachkov, E. Cayani, F. S. Bartlett III, W. N. Frankel, et al. 1997. TRANCE is a novel ligand of the tumor necrosis factor receptor family that activates c-Jun N-terminal kinase in T cells. *J. Biol. Chem. 272:25190*.
58. Liu, C.-C., B. Perussia, Z. A. Cohn, and J. D.-E. Young. 1986. Identification and characterization of a pore-forming protein of human peripheral blood natural killer cells. *J. Exp. Med. 164:2061*.
59. Gershenfeld, H. K., R. J. Herschberger, T. B. Shows, and I. L. Weissman. 1988. Cloning and chromosomal assignment of a human cDNA encoding a T cell- and natural killer cell-specific trypsin-like serine protease. *Proc. Natl. Acad. Sci. USA 85:1184*.
60. Trapani, J. A., J. L. Klein, P. C. White, and B. Dupont. 1988. Molecular cloning of an inducible serine esterase gene from human cytotoxic lymphocytes. *Proc. Natl. Acad. Sci. USA 85:6924*.
61. Salcedo, T. W., L. Azzoni, S. F. Wolf, and B. Perussia. 1993. Modulation of perforin and granzyme messenger RNA expression in human natural killer cells. *J. Immunol. 151:2511*.
62. Arase, H., N. Arase, and T. Saito. 1995. Fas-mediated cytotoxicity by freshly isolated natural killer cells. *J. Exp. Med. 181:1235*.
63. Liu, C., S. Rafii, A. Graniello-Piperno, J. A. Trapani, and J. D-E. Young. 1989. Perforin and serine esterase gene expression in stimulated human T cells: kinetics, mitogen requirements, and effects of cyclosporin A. *J. Exp. Med. 170:2105*.
64. Suda, T., T. Okazaki, Y. Naito, T. Yokota, N. Arai, K. Ozaki, and S. Nagata. 1995. Expression of Fas ligand in cells of T cell lineage. *J. Immunol. 154:3806*.
65. Stalder, T., S. Hahn, and P. Erb. 1994. Fas antigen is the major target molecule for $CD4^+$ T cell-mediated cytotoxicity. *J. Immunol. 152:1127*.
66. Ju, S., H. Cui, D. J. Panka, R. Ettinger, and A. Marshak-Rothstein. 1994. Participation of target Fas protein in apoptosis pathway induced by $CD4^+$ Th1 and $CD8^+$ cytotoxic T cells. *Proc. Natl. Acad. Sci. USA 91:4185*.
67. Nastala, C. L., H. D. Edington, T. G. McKinney, H. Tahara, M. A. Nalesnik, M. J. Brunda, M. K. Gately, S. F. Wolf, R. D. Schreiber, W. J. Storkus, et al. 1994. Recombinant IL-12 administration induces tumor regression in association with IFN-γ production. *J. Immunol. 153:1697*.
68. Luster, A. D., S. M. Greenberg, and P. Leder. 1995. The IP-10 chemokine binds to a specific cell surface heparan sulfate site shared with platelet factor 4 and inhibits endothelial cell proliferation. *J. Exp. Med. 182:219*.
69. Angiolillo, A. L., C. Sgadari, D. D. Taub, F. Liao, J. M. Farber, S. Maheshwari, H. K. Kleinman, G. H. Reaman, and G. Tosato. 1995. Human interferon-inducible protein 10 is a potent inhibitor of angiogenesis. *J. Exp. Med. 182:155*.
70. Ghosh, P., K. L. Komschlies, M. Cippitelli, D. L. Longo, J. Subleski, J. Ye, A. Sica, H. A. Young, R. H. Wiltrout, A. C. Ochoa. 1995. Gradual loss of T-helper 1 populations in spleen of mice during progressive tumor growth. *J. Natl. Cancer Inst. 87:1478*.
71. Mulders, P., R. Figlin, J. B. DeKernion, R. H. Wiltrout, M. Linehan, D. Parkinson, W. deWolf, and A. Belldegrun. 1997. Renal cell carcinoma: recent progress and future directions. *Cancer Res. 57:5189*.